creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

치의학박사 학위논문

Effects of triethylene glycol dimethacrylate and hydroxyethyl methacrylate on macrophage polarization

Triethylene glycol dimethacrylate와
hydroxyethyl methacrylate가
대식세포의 분극에 미치는 영향

2019년 8월

서울대학교 대학원
치의과학과 치과생체재료과학 전공
김 인 숙

Effects of triethylene glycol dimethacrylate and hydroxyethyl methacrylate on macrophage polarization

지도교수 양 형 철

이 논문을 치의학박사 학위논문으로 제출함

2019년 5월

서울대학교 대학원

치의과학과 치과생체재료과학 전공

김 인 숙

김인숙의 박사 학위논문을 인준함

2019년 7월

위 원 장 임 범 순 (인)

부위원장 양 형 철 (인)

위 원 권 일 근 (인)

위 원 안 진 수 (인)

위 원 김 선 영 (인)

-ABSTRACT-

Effects of triethylene glycol dimethacrylate and hydroxyethyl methacrylate on macrophage polarization

In-Sook Kim, D.D.S., M.S.

Department of Dental Biomaterials Science

School of Dentistry, Seoul National University

(Directed by professor **Hyeong-Cheol Yang**, Ph.D)

The effects of dental resin monomers, triethylene glycol dimethacrylate (TEGDMA) and hydroxyethyl methacrylate (HEMA), on the polarization of a human monocyte cell line (THP-1) were evaluated.

THP-1 cells were treated with resin monomers at non-cytotoxic concentrations for 48 h and were analyzed for CD86 and CD206 expressions using flow cytometry. The cells were stimulated for

polarization in the presence of resin monomers (co-treatment), or after treatment with monomers (pre-treatment). CD86 and CD206 mRNA in co-treated cells were evaluated using quantitative real-time polymerase chain reaction. The release of TNF-α and TGF-β by pre-treated and co-treated cells was assessed using enzyme-linked immunosorbent assay. Morphological changes of macrophages during polarization were observed using bright-field microscopy. One-way analysis of variance was used for statistical analysis.

TEGDMA (1 mmol/L) and HEMA (2 mmol/L) did not induce CD86 and CD206 expressions in THP-1 cells but rather inhibited their expressions in the co-treated cells. The inhibitory effects also appeared at the transcription level. However, the expression of surface markers was not affected by pre-treatment with resin monomers. The release of TNF-α and TGF-β by M1- and M2-stimulated cells, respectively, was suppressed by co-treatment ($P<0.05$). Microscopic studies revealed that co-treatment with resin monomers suppressed polarization-associated morphological changes such as cell volume increase.

In conclusion, TEGDMA and HEMA inhibited macrophage polarization to both M1 and M2 at the transcription level, and the inhibitory effects disappeared upon the removal of resin monomers from the cell culture.

Key words: hydroxyethyl methacrylate, triethylene glycol dimethacrylate, macrophage, polarization

Student Number: 2011 - 31194

CONTENTS

LIST OF FIGURES

I. Introduction

Dental resin monomers can leach out from resin-based dental materials, such as resin composites and dentine bonding agents, interacting with the cellular components of the dental pulp, eventually inducing adverse effects on the biological processes within tissues. Cytotoxicity and its underlying mechanism are the most studied biological effects of resin monomers. Triethylene glycol dimethacrylate (TEGDMA) was reported to reduce the viability of human pulp cells in a dose-dependent manner at concentrations of 0.1 - 3 mmol/L [1]. The cytotoxicity of TEGDMA has been attributed to a drastic depletion of intracellular glutathione, followed by the occurrence of excessive reactive oxygen species (ROS), which could undermine cell viability. The role of ROS in TEGDMA-induced cell damage was indirectly revealed by the antagonistic effect of antioxidants on the cytotoxicity of resin monomers [1, 2]. Hydroxyethyl methacrylate (HEMA), hydrophilic dental resin monomer, was also reported to deplete glutathione and produce excessive ROS [3], and the antioxidant N-acetylcysteine (NAC) was reported to reduce HEMA-derived ROS and cell death [4]. These results indicated that oxidative stress was involved in HEMA cytotoxicity. Moreover, the mutagenicity of resin monomers has been demonstrated by *in vitro* micronucleus studies [5, 6]. Because NAC attenuated the mutagenicity of TEGDMA and HEMA, it was suggested that oxidative stress mediated the mutagenic and cytotoxic effects of the resin monomers.

At high concentrations, resin monomers can cause

cytotoxicity-derived inflammation; necrotic cells release pro-inflammatory cellular components into adjacent cells and tissues. Previous studies have shown that inflammation could be provoked by the monomers, even at sub-lethal concentrations [7, 8]. It was shown that TEGDMA and HEMA enhanced the mRNA expression of cyclooxygenase-2 (COX-2) gene, a key enzyme in tissue inflammation, in a mouse macrophage cell line and dental pulp cells, and that TEGDMA promoted the synthesis of prostaglandin E2, an inflammation mediator. Therefore, it was speculated that dental resin monomers might induce inflammation of the dental pulp and oral mucosa when they are released from resin-based bonding agents and restorative composites. In contradiction to the pro-inflammatory potency of resin monomers, TEGDMA and HEMA inhibited the release of inflammatory cytokines such as IL-1β and TNF-α in lipopolysaccharide (LPS)-exposed macrophages [9, 10]. The inhibitory effects of monomers on these cytokines showed that resin monomers would suppress the inflammatory response of macrophages to bacteria.

Pulpal inflammatory regions associated with dental caries are heavily populated with immunocompetent macrophages [11, 12]. Those macrophages at the infected regions induce inflammation to defend tissues against pathogens and were categorized as pro-inflammatory macrophages, M1. Macrophages were known to polarize into two phenotypes, M1 and M2 [13]. The M1 phenotype is induced by LPS and interferon (IFN)-ɣ, and promotes inflammation by releasing

pro-inflammatory cytokines such as IL-1β and TNF-α. The M2 phenotype secretes anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β, resolving inflammation and promoting tissue regeneration. In the healing of mineral trioxide aggregate-capped pulp tissue, M2 macrophages transiently accumulated beneath the provisional layer of regeneration, which suggested that M2 macrophages were involved in the healing of pulp tissues [14]. The role of M2 macrophages in pulpal healing was further reported in another study in which M2 macrophages enhanced the odontogenic differentiation of dental pulp stem cells [15]. Therefore, both M1 and M2 macrophages are expected to play a role in the inflammation and regeneration of the pulp tissues of damaged teeth, where they may be exposed to dental resin monomers following teeth restoration with resin-based materials. Many studies have been performed about cellular and genetic toxicity of dental resin monomers and these previous studies have used mainly human or mouse pulp cells to determine the cytotoxicity of resin monomers. By the way, recently some researchers used human monocyte cell lines to evaluate the biological effects of resin based materials. Heil *et al.* compared the 50% cytotoxicity values and TNF-α secretion using the peripheral blood monocytes and THP-1 cell [16]. However, the effects of resin monomers on macrophage polarization have not been thoroughly investigated. Therefore in the present study, the effects of TEGDMA and HEMA on macrophage polarization to M1 and M2 was observed using flow cytometry analysis of cell surface markers and

microscopic observation of morphological changes. The release of TNF-α and TGF-β in resin monomer-treated M1 and M2 macrophages was also quantified. Additionally we observed the effects of resin monomers on ATP level.

II. Literature Review

1. Biological effects of resin monomers.

Dental resins have been used as direct/indirect filling materials, dentin bonding agents to treat the defective teeth and as a part of the fixed or removable prostheses [17, 18]. Generally these materials are cured by polymerization process after the placement in the tooth. In a certain clinical condition, resin monomers could be released from incompletely cured resin materials into oral environment [17, 19]. The released monomers can reach to pulp tissue through dentin and cause cytotoxicity. TEGDMA can easily pass through the cell membrane and rapidly react with intracellular glutathione (TEGDMA-GSH) resulting in decrease of intracellular detoxifying energy [20-22]. It was reported that TEGDMA inhibited cell proliferation and reduced cell viability, furthermore induced apoptosis or necrosis on THP-1 monocytes [17]. HEMA also has been known to influence toxic effects on mammalian cells *in vitro* studies. HEMA-GSH covalent binding caused oxidative stress by increasing free radicals or peroxides [23, 24]. Nocca *et al.* reported that HEMA did not change viability and proliferation but affect cellular differentiation, oxygen consumption and GSH level in HL-60 cells at 1.1 mmol/L for 24 and 48 h [25]. Monomer-induced ROS may cause genotoxicity and apoptosis. ROS interact with pyrimidines and purines as well as sugar and chromatin proteins of DNA. Consequently ROS-DNA brings about double-strand breaks, DNA base modifications and

protein cross-links [26]. Also double-strand breaks leads to activation of kinase signaling cascades which reduce the genome progression and mediate DNA-repair pathways. Activated ataxia telangiectasia mutated (ATM) gene detects cell-cycle arrest in the S-phase, and targets cellular check-point kinase Chk2, and histon H2AX followed by phosphorylation of p53 which induces transcription of pro-apoptotic proteins [27]. It was revealed that TEGDMA caused systemic toxicity by *in vitro* and *in vivo* studies. This diluent monomer can be rapidly permeated into aqueous oral environment and consequently distributed systemically. Systemic cytotoxic effects can be identified by investigating acute inhalation toxicity, alterations of systemic organs, cardiovascular effects and acute oral toxicity [28]. ED_{50} values of TEGDMA were 0.12-0.26 mM in primary human pulpal fibroblasts, and 1.4 mM in JTC-12 epithelial (monkey kidney) cells. These concentrations is relatively higher than other aqueous monomers' values [28, 22]. Also *in vivo* research has been reported about the systemic cytotoxicities of TEGDMA. Reichl *et al.* measured the amounts of ^{14}C-TEGDMA in various organs such as heart, stomach, muscle, and spleen of guinea pigs and mice. They found that ^{14}C-TEGDMA widely distributed from brain to small intestine, but fortunately were not detected in the examined tissues after 24 h because it was excreted rapidly after administration [29]. Allergic effects of dental materials were variously investigated *in vivo* especially in dental personnel studies. Delayed hypersensitivity to HEMA-containing primers in guinea pigs, long-standing sensitizing

reactions to methacrylates (dentures) and systemic adverse symptoms (whole-body urticaria, bronchospasm, blistering of the face, etc) to TEGDMA based sealant in dental patients have been previously described [30-32]. Dental hygienists were reported to be hypersensitivity to glutaraldehyde and benzoyl peroxide whereas dentists showed allergic reactions to HEMA, MMA, and TEGDMA [28]. Among these monomers, HEMA was the strongest sensitizer causing paresthesia of the fingertips and allergic pharyntitis in patch tests [33].

2. Inflammation and regeneration in dental pulp

Dental caries, trauma and chemical agents such as dental resin monomers can evolve the inflammatory or regenerative responses by cellular and molecular signaling in the dentin-pulp tissue [34]. At relatively early infection stage, tubular odontoblasts involved in reactionary dentinogenesis. Because caries bacteria and monomers diffuse into dentinal tubule, tubular odontoblasts which lining the pulp chamber are the first cells to regulate an inflammatory responses. They are able to detect foreign invader, release cytokines and antimicrobial peptides to resolve the infection [35]. As tissue injury progresses, pulp fibroblasts, stem cells, and innate immune cells are recruited at the infection site to form a secretory tertiary dentin above the exposed pulp [36]. As well as hard tissue restoration, soft pulpal tissue, angiogenic and neurogenic tissue will be repaired [37]. Vascular endothelial growth factor (VEGF), nerve growth factor (NGF) and several pro-inflammatory cytokines contribute to the these

regenerative processes [35]. Pattern recognition receptors, nuclear transducers and transcription factors are involved in molecular signaling network. TLRs expressed on the immunocompetent cells detect the irritants and bind their ligands to activate the NF-κB, p38, MAP kinase which lead to secrete the extracellular cytokines and chemokines [38]. TNF-α, TGF-β, IL-4, IL-6, and etc. are key mediators triggering the cellular signal cascades and resulting in tissue repair. At high concentrations, resin monomers can cause cytotoxicity-derived inflammation; necrotic cells release pro-inflammatory cellular components into adjacent cells and tissue. Previous studies have shown that inflammation could be provoked by the monomers, even at sub-lethal concentrations [7, 8]. It was shown that TEGDMA and HEMA enhanced the mRNA expression of cyclooxygenase-2 (COX-2) gene, a key enzyme resulting in tissue inflammation, in a mouse macrophage cell line and dental pulp cells, and that TEGDMA promoted the synthesis of prostaglandin E2, an inflammation mediator. Therefore, it was speculated that dental resin monomers might induce inflammation of the dental pulp and oral mucosa when they are released from resin-based bonding agents and restorative composites. In contradiction to the pro-inflammatory potency of resin monomers, TEGDMA and HEMA inhibited the release of inflammatory cytokines such as IL-1β and TNF-α in LPS-exposed macrophages [9, 10]. The inhibitory effects of monomers on these cytokines showed that resin monomers would suppress the inflammatory response of macrophages to bacteria.

3. Polarization of macrophages

Monocyetes and macrophages are the critical immune cells which initiate and orchestrate inflammatory responses [39]. Monocytes circulate through blood, bone marrow, nonhematopoietic organs and differentiate to tissue macrophages depending on the stimuli in infection site. Tissue macrophages can be replicated, differentiated for enhancing cytotoxic, inhibitory activities to resolve inflammation and tissue remodeling [40].

Pulpal inflammatory regions associated with dental caries are heavily populated with immunocompetent macrophages [11, 12]. Those macrophages at the infected regions induce inflammation to defend tissues against pathogens and were categorized as pro-inflammatory macrophages, M1. Macrophages can be divided into two main classes according to their activation phenotypes [41] associated with microbicidal activity and surface antigen presenting. When macrophages are exposed to IFN-ɣ and/or lipopolysaccharide (LPS), they are polarized into M1 [42], while exposure to interleukin-4 (IL-4) or interleukin-13 (IL-13) polarize the macrophages into M2 [43]. M1 surface markers are CD 86, CD 25 and MHC class II molecules whereas M2-associated markers include CD 206, CD 209, SR-A1, and etc. M1 polarized macrophages produce pro-inflammatory cytokines including C-X-C motif ligand 10, TNF-α, IL-1β, IL-6 and reactive oxygen species infiltrating into injured tissues [44] for initiating and facilitating innate immune reaction and wound debridement. The M2 phenotype secretes anti-inflammatory cytokines

such as IL-4, IL-10, and TGF-β, resolving inflammation and promoting tissue regeneration. In the healing of mineral trioxide aggregate-capped pulp tissue, M2 macrophages transiently accumulated beneath the provisional layer of regeneration, which suggested that M2 macrophages were involved in the healing of pulp tissues [14]. The role of M2 macrophages in pulpal healing was further reported in another study in which M2 macrophages enhanced the odontogenic differentiation of dental pulp stem cells [15]. Therefore, both M1 and M2 macrophages are expected to play a role in the inflammation and regeneration of the pulp tissues of damaged teeth, where they may be exposed to dental resin monomers following teeth restoration with resin-based materials.

III. Materials and methods

1. Cell culture and polarization of THP-1

THP-1, a human monocyte cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) and 100 U/mL penicillin G at 37°C in a humidified atmosphere (5% CO_2, 95% O_2). Macrophage polarization was performed according to a previously established method [45]. Briefly, THP-1 cells were plated in a 6-well plate ($2x10^5$ cells/well), and differentiated to the pre-active M0 state by incubation with 100 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) for 2 days, followed by 2 days without PMA. Then, the M0 macrophages were polarized to the M1 phenotype by exposure to 40 ng/mL LPS and 40 ng/mL IFN-γ for two days, and M2 macrophages were prepared by treatment with IL-4 (40 ng/mL).

2. Cytotoxicity assessment

The cytotoxicity of TEGDMA and HEMA (Sigma-Aldrich, St Louis, MO, USA) was assessed with THP-1 cells induced to the M0 state by PMA exposure for 2 days. Thereafter, the cells were washed with phosphate-buffered saline (PBS, pH 7.4) and resuspended in fresh culture media in a 96-well plate containing TEGDMA and HEMA at

concentrations of 0.5 - 8.0 mmol/L. After 2 days, cell viability was determined according to a previously established protocol [46]. Briefly, the cells were washed and replenished with culture media containing 10%2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)5-(2,4-disulfophenyl)-2H-tetrazoliumm (Dojindo Laboratories, Kumamoto, Japan) for 1 h at 37°C. The optical density of the colored reactants was measured using a Tecan Sunrise™ absorbance microplate reader (Tecan, Salzburg, Austria).

3. Resin monomer treatment and flow cytometry analysis

The effects of resin monomers on macrophage polarization were investigated under three different conditions: 1) resin monomer treatment only, 2) co-treatment with the polarization-stimulating reagents, and 3) pre-treatment followed by exposure to the polarization-stimulating reagents. For the resin monomer treatment only, THP-1 cells at the M0 state were exposed to TEGDMA (1 mmol/L) and HEMA (2 mmol/L) for 2 days and then analyzed for CD86 and CD206 expressions using a fluorescence-activated cell sorting (FACS). For the co-treatment group, the M0 macrophages were exposed to M1-inducing agents (LPS and IFN-ɣ) or an M2-inducing agent (IL-4), with or without resin monomers for 2 days. For the pre-treatment group, PMA-treated macrophages were incubated with TEGDMA and HEMA for 2 days, washed with PBS, and then exposed to the M1- and M2-inducing agents for 2 days,

followed by the analysis of CD86 and CD206 expressions using FACS. The macrophage polarization state was determined using flow cytometric analysis of CD86 and CD206 surface maker proteins to identify the M1 and M2 phenotypes, respectively. The macrophages were incubated with fluorescein isothiocyanate-conjugated anti-human mouse antibody (R&D Systems, Minneapolis, MN, USA), and their fluorescence was analyzed using a FACSCalibur™ flow cytometer (Becton-Dickinson, San Jose, CA, USA).

4. Cytokine analysis

The release of TNF-α and TGF-β by THP-1 cells was observed using ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). The sample signals were detected at 450 nm using a plate reader (Tecan).

5. Gene expression assay

To investigate the effects of resin monomers on macrophage polarization at the transcription level, gene expression of CD86 and CD206 was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). The primer pairs were as follows: CD86, forward 5′-ATGGGACTGAGTAACATTCTCTTTGTG ATGGCCT-3′, reverse 5′-CTCGAGTTAAAAACATGTATC ACTTTTGTCGC ATGA-3′; CD206, forward 5′-TTCGGACACCCATCGGAAT TT-3′, reverse 5′-CACAAGCGCTGCGTGGAT-3′; and glyceraldehyde 3-phosphate dehydrogenase, forward 5′

-GTCGGAGTCAACGGATTTGG-3′, reverse 5′ -GGGTGGAATCATATTGGAACATG-3′. The PCR conditions were 95°C for 30s followed by 40 cycles of denaturation at 95°C for 15s and annealing at 60°C for 34s. The gene expression of CD86 and CD206 was normalized to that of the reference gene glyceraldehyde 3-phosphate dehydrogenase.

6. ATP assay

The effects of resin monomers on ATP level was observed. THP-1 cells of M0 state were exposed to TEGDMA (1 mmol/L) and HEMA (2 mmol/L) for 24 h, and the amount of ATP was measured using a colorimetric ATP quantitation kit according to the manufacturer's instructions (EZ-ATP Assay Kit, DoGenBio Co. Ltd., Seoul, Korea). 10% DMSO-treated cells were used as non-viable control macrophages.

7. Statistical analysis

Statistical analyses were performed using the SPSS 22 statistical software (SPSS, Chicago, IL, USA). Differences among groups in the ELISA and qRT-PCR experiments were analyzed by one-way analysis of variance followed by Tukey's test. P-values of <0.05 were considered statistically significant.

IV. Results

1. Cytotoxic activity of resin monomers on THP-1 cells

TEGDMA and HEMA cytotoxicities were assessed to determine the highest non-cytotoxic concentrations for THP-1 cells, and cell viability decreased significantly at 2 mmol/L TEGDMA and 4 mmol/L HEMA (Fig. 1). Thus, TEGDMA and HEMA were applied at 1 and 2 mmol/L, respectively, in further experiments of macrophage polarization in this study.

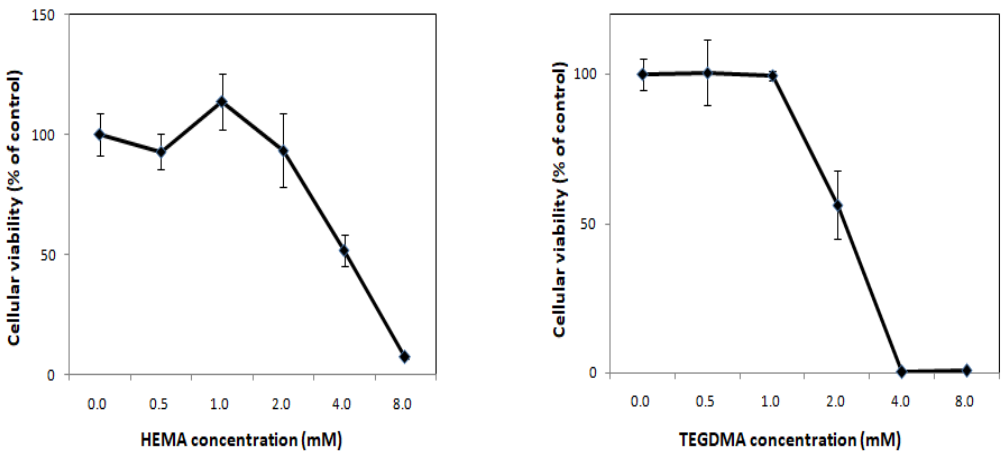

Figure 1. Cytotoxic effects of HEMA and TEGDMA on THP-1 M0 macrophage.

Cells were treated with various concentrations of HEMA and TEGDMA for 48 h and then viability was evaluated by WST-8 assay. Each data point represents the mean and standard deviation of three independent experiments per each condition and is expressed as a percentage values of the respective control group.

2. Flow cytometry analysis

2.1 Effects of resin monomers on macrophage polarization

TEGDMA- and HEMA-treated THP-1 cells were analyzed for CD86 (M1 surface marker) and CD206 (M2 surface marker) expressions (Fig. 2) to investigate whether TEGDMA and HEMA were able to polarize macrophages into M1 and M2. Most of the M1 and M2 cells were CD86 positive (96.2%) and CD206 positive (99.2%), respectively (Fig. 2C, F), while M0-state cells did not express either M1 or M2 markers (Fig. 2A, B). TEGDMA and HEMA did not alter CD86 and CD206 expressions in M0 macrophages, which indicates that the resin monomers did not induce macrophage polarization.

Figure 2. Effects of TEGDMA and HEMA on CD86 and CD206 expressions. THP-1 cells were treated with 1 mmol/L TEGDMA and 2 mmol/L HEMA for 48 h, and then cell surface markers CD86 and CD206 were labeled with FITC-conjugated antibody for FACS analysis. M0 (A, B), M1 (C, D), and M2 (E, F) phenotypes were induced and used as controls.

2.2. Effects of resin monomers on the induction of macrophage polarization

THP-1 cells were exposed to LPS/IFN-ɣ or IL-4 to induce phenotype polarization in the presence of resin monomers. The shift of CD86 by LPS/IFN-ɣ was almost entirely inhibited by TEGDMA and HEMA (Fig. 3C, G). Resin monomers also suppressed the IL-4-induced change of CD206 expressions (Fig. 3F, J) and did not affect CD86 and CD206 expressions during M1 and M2 polarization, respectively (Fig. 3D, E, H, J). Therefore, it is certain that TEGDMA and HEMA strongly blocked macrophages polarization to both M1 and M2. The persistence of the blocking effect was investigated by pre-treatment with resin monomers before polarization induction (Fig. 4). TEGDMA pre-treated cells expressed CD86 and CD206 under polarization inducing conditions (Fig. 4C, F). HEMA-pre-treated cells expressed CD86 and CD206 upon M1 and M2 induction, respectively (Fig. 4G, J).

Figure 3. Effects of TEGDMA and HEMA on CD86 and CD206 expressions during M1 and M2 phenotype induction, respectively. THP-1 cells at the M0 state were treated with polarization agents in the presence of TEGDMA (C - F) and HEMA (G - J). After M1 (C, D, G, H) and M2 (E, F, I, J) activation for 48 h, CD86 and CD206 were labeled with FITC-conjugated antibody for FACS analysis.

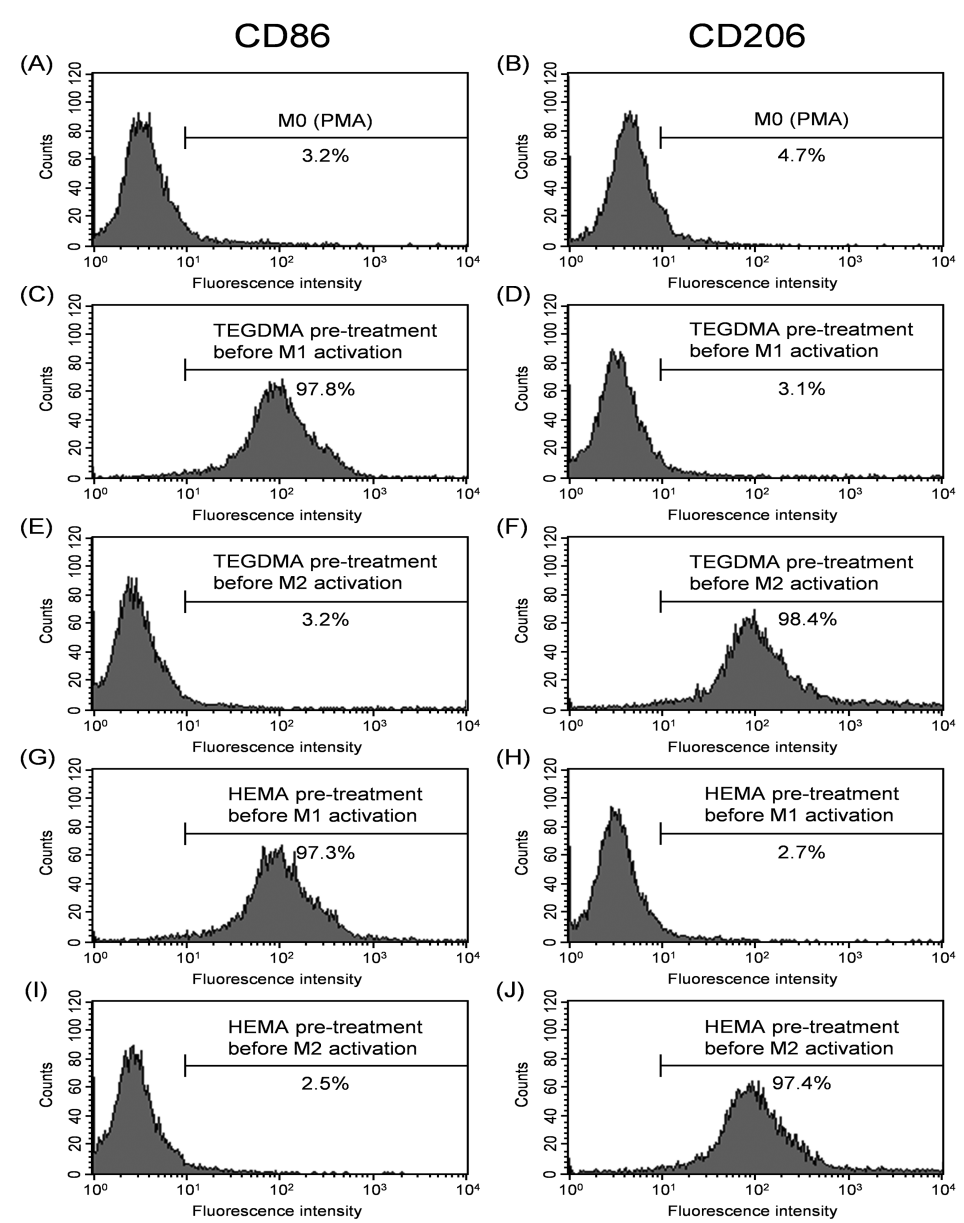

Figure 4. Effects of resin monomer pre-treatment on CD86 and CD206 expressions. THP-1 cells were pre-treated with TEGDMA (C - F) and HEMA (G - J) for 48 h, washed, and then exposed to M1- and M2-inducing agents for 48 h.

3. TNF-α and TGF-β release by resin monomer-treated cells

The release of TNF-α and TGF-β in the culture media of THP-1 cells was assessed to investigate the effects of resin monomers on the expression of polarization-related growth factors. The release of TNF-α was completely dependent on the presence of M1-inducing agents, which increased the amount of TNF-α from 5 to 1732 pg/mL (Fig. 5A, B). When the M1-inducing agents were removed from the culture media, the release of TNF-α was down regulated to an almost negligible level of 30 pg/mL. Pre-treatment of cells with resin monomers followed by M1 induction without the monomers did not affect the release pattern of TNF-α ; in other words, secretion was induced in the presence of M1-inducing agents. However, TNF-α release by M1 induction was largely prevented when the cells were exposed to TEGDMA and HEMA, which was consistent with the results of CD86.

THP-1 cells produced TGF-β in response to IL-4 (Fig. 5C, D). TGF-β levels increased from 865 to 2086 pg/ml at the M0 state afte r the treatment with IL-4. The TGF-β concentrations (Fig. 5C, D) were calculated after subtracting the background serum level. Contrary to TNF-α, the release of TGF-β continued even in the absence of IL-4, and 1994 pg/mL of TGF-β was detected in M2-induced THP-1 cells when IL-4 was removed from the culture. The effects of TEGDMA and HEMA on the release of TGF-β were similar to that of TNF-α. Pre-treatment with resin monomers did not

affect the release pattern of TGF-β, and IL-4 did not induce an increase in TGF-β secretion in the presence of TEGDMA and HEMA.

Figure 5. Effects of TEGDMA and HEMA on the release of TNF-α and TGF-β by THP-1 cells. For the analysis of TNF-α of M1-induced cells (A, B) and TGF-β of M2-induced cells, THP-1 cells were pre-treated or co-treated with resin monomers. TNF-α and TGF-β were measured at three stages: 1) before M1 or M2 activation (pre-activation), 2) just after activation for 48 h (M1-activated, M2-activated), and 3) at 48 h after removal of the polarization reagents (post-activation). Error bars represent the mean value and standard deviation obtained from three independent experiments. * indicates statistically significant differences ($p<0.05$).

4. mRNA expression of CD86 and CD206 in resin monomer-treated cells

The effects of resin monomers on the mRNA expression of polarization marker genes were observed. THP-1 cells were co-treated with resin monomers and polarization-inducing agents, and then, the transcripts of CD86 and CD206 were quantitated. The M1-inducing LPS/IFN-γ remarkably increased the amount of CD86 mRNA, while the increase was blocked by TEGDMA and HEMA nearly to the level at the M0 state (Fig. 6A). The induction of CD206 mRNA was also suppressed by resin monomers in M2-induced THP-1 cells (Fig. 6B).

Figure 6. CD86 and CD206 mRNA expression of M1 and M2 macrophages. THP-1 cells were activated with M1- and M2-inducing agents in the presence of TEGDMA and HEMA. CD86 (A) and CD206 (B) mRNA expression was quantitated for M1- and M2-activated cells, respectively. Error bars represent the mean value and standard deviation obtained from three independent experiments. * indicates statistically significant differences from the PMA group ($p<0.05$).

5. Effects of resin monomers on polarization-induced morphological changes

Morphological responses of THP-1 to M1 and M2 induction in the presence of TEGDMA and HEMA was observed. Untreated cells were small and round, exhibiting typical monocyte morphology (Fig. 7A). Upon exposure to PMA, THP-1 cells became larger and irregularly shaped (Fig. 7B). M1 macrophages also enlarged (2 - 3 times larger than the untreated group) and irregularly shaped (Fig. 7C) following exposure to M1-inducing agents. Similarly, M2 macrophages (Fig. 7D) contained protrusions and were elongated or spread out following exposure to M2-inducing agents. However, when M1 and M2 induction was performed in the presence of resin monomers, the cells were round and did not exhibit morphological features of the M1 and M2 phenotypes (Fig. 7E - H).

Figure 7. Microscopic photographs of THP-1 cells. Inactive THP-1 cells (A) were treated with PMA (B) and then exposed to M1-inducing agents (C, E, G) and M2-inducing agents (D, F, H) in the presence of TEGDMA (E, F) and HEMA (G, H). Photographs were captured under low (100×) and high magnification (400×).

6. ATP levels of resin monomer-treated cells

The ATP levels of resin monomer-treated cells were evaluated to investigate the relevance of mitochondrial function in macrophage polarization. As shown in Figure 7, TEGDMA and HEMA did not induce changes in APT levels. Nearly all ATP were lost in 10% DMSO cells, the non-viable control macrophages.

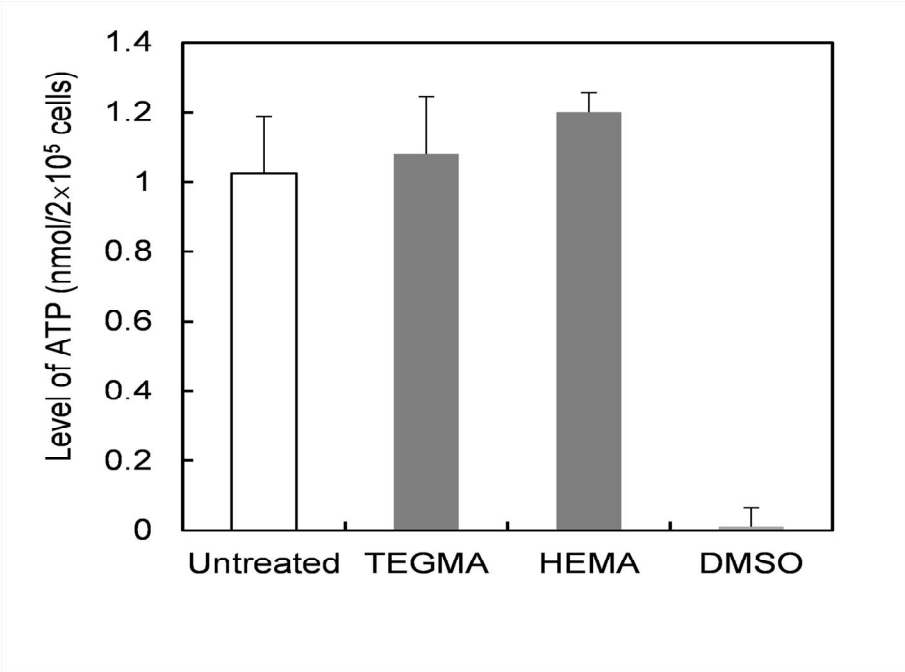

Figure 8. ATP levels of THP-1 cells. PMA-treated cells (M0 state) were exposed to TEGDMA, HEMA, and 10% DMSO for 24 h. Error bars represent the mean value and standard deviation obtained from three independent experiments. There were no statistically significant differences in ATP level among untreated, TEGDMA, and HEMA groups.

V. Discussion

In addition to the critical function of macrophages in the immune response to foreign materials, macrophages play a role in various biological processes such as tissue patterning and branching morphogenesis in organ development, angiogenesis, stem cell differentiation and erythropoiesis [47-50]. In the dental pulp of healthy teeth, a large number of immunocompetent macrophages have been found [51]. Considering the multifunctionality and the presence of macrophages in the dental pulp, the immune cells are expected to play a role in tooth development and regeneration as well as in defence against caries-causing pathogens, although direct evidence of physiological functions in healthy teeth is limited. However, because macrophages are necessarily involved in the wound healing of tissues, macrophages likely affect the regeneration of injured dental pulp and dentine. Therefore, the factors that influence the phenotype and function of macrophages may consequently affect pulp wound healing. In this study, three kinds of protocols for resin monomer treatment were employed: (i) treatment with resin monomers alone, (ii) co-treatment of resin monomers and polarization-stimulating agents and (iii) pre-treatment with resin monomers before the exposure to polarization-stimulating reagents. Treatment with resin monomers alone was performed to determine whether resin monomers were able to induce macrophage polarization. The co-treatment protocol was used to examine the effect of resin monomers on the induction of macrophage polarization. Finally, the pre-treatment test was carried

out to investigate the reversibility of resin monomer effect. The results of those experiments showed that TEGDMA and HEMA inhibited the induction of M1 and M2 phenotypes, of which effect was reversible.

M1 and M2 macrophages play important respective roles from the occurrence of injury to the end of healing. Pro-inflammatory M1 macrophages defend tissues via immune reaction enhancement and engulfment of pathogens and dead cells. If M1 macrophages are not induced, the infected tissue may lose the ability to fight against pathogens, resulting in more severe and even irreversible damage. Previous studies concerning the effects of resin monomers on macrophage functions have mainly focused on the release of inflammatory cytokines by LPS-treated cells [9, 10, 52]. These studies demonstrated the inhibitory effects of TEGDMA and HEMA on the secretion of TNF-α and IL-1β from LPS-treated RAW264.7, a murine macrophage cell line. The expression of surface antigens in LPS-treated cells was also suppressed by TEGDMA [9]. These results suggested that resin monomers interfered with the phenotype shift of macrophages to pro-inflammatory M1 cells. In this study, the effects of resin monomers on the phenotype shift to M2 as well as M1 macrophages were observed. The shift to both M1 and M2 was entirely blocked by TEGDMA and HEMA (Fig. 3), and the blocking effects appeared at the transcriptional level (Fig. 6). Therefore, the resin monomers likely affected the polarization and function of macrophages across a broad range of mechanisms, although the

molecular mechanism that underlies these inhibitory effects is not yet elucidated. The inhibitory effects of TEGDMA and HEMA on macrophage polarization were quite substantial (Figs 3 and 5). However, the effects disappeared completely when the monomers were removed from the culture (Figs 4 and 5), indicating that the influence of resin monomers is temporary and reversible. Therefore, the retention period of resin monomers inside the pulp cavity may determine the severity of the effects on macrophage polarization in clinical circumstances. If dental resin monomers remain in the pulp longer than the duration of wound healing, pulp tissue recovery and dentine regeneration are expected to be interfered with or delayed.

In this study, the underlining mechanism for the inhibition of macrophage polarization was not investigated. The most well-known effects of resin monomers on mammalian cells are the depletion of intracellular glutathione and generation of ROS, which eventually causes oxidative stress [3]. ROS production is critical for M1 activation, and it was recently determined that ROS was also necessary for M2 macrophage differentiation [53]. Therefore, oxidative stress does not seem to be involved in the resin monomer's inhibitory effects on macrophage polarization. Except for the occurrence of ROS, the glutathione depletion can cause another serious cellular abnormality, the loss of ATP. A previous study demonstrated that the depletion of glutathione was rapidly followed by ATP loss [54]. ATP loss can affect a broad range of cellular activity such as macrophage polarization. However, the level of ATP was not altered

by TEGDMA and HEMA in the experiments (Fig. 8). The difference in the results may come from monomer concentrations, treated cell numbers and cell types. It is also possible that the level of ATP was temporarily downregulated by resin monomers and recovered before ATP analysis. In that case, the temporary shortage of ATP could inhibit macrophage polarization. Overall, the results do not provide any evidence regarding the involvement of mitochondrial defects in the failure of macrophage polarization. However, the relationship cannot be completely denied, unless a clear cause of resin monomer effects on macrophages is revealed. The effects of resin monomers on macrophages undergoing polarization were observed. The monomers may also encounter already-polarized macrophages in the dental pulp. Therefore, it is necessary to investigate the impact of dental resin monomers on the function of fully polarized M1 and M2 macrophages in future studies.

VI. Conclusions

TEGDMA and HEMA inhibited macrophage polarization to M1 and M2 phenotypes and polarization-associated cytokine release. This inhibitory effect was shown at the transcription level. The ability of macrophages to polarize was revived by the removal of resin monomers from the cell culture.

References

[1] Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Périanin A. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J Biomed Mater Res A. 2003;66:476-82.

[2] Walther U, Siagian, II, Walther SC, Reichl FX, Hickel R. Antioxidative vitamins decrease cytotoxicity of HEMA and TEGDMA in cultured cell lines. Arch Oral Biol. 2004;49:125-31.

[3] Chang HH, Guo MK, Kasten FH, Chang MC, Huang GF, Wang YL, Wang RS, Jeng JH. Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. Biomaterials. 2005;26:745-53.

[4] Spagnuolo G, D'Anto V, Cosentino C, Schmalz G, Schweikl H, Rengo S. Effect of N-acetyl-L-cysteine on ROS production and cell death caused by HEMA in human primary gingival fibroblasts. Biomaterials. 2006;27:1803-9.

[5] Lee DH, Lim BS, Lee YK, Ahn SJ, Yang HC. Involvement of oxidative stress in mutagenicity and apoptosis caused by dental resin monomers in cell cultures. Dent Mater. 2006;22:1086-92.

[6] Schweik H, Hartmann A, Hiller KA, Spagnuolo G, Bolay C, Brockhoff G, Schmalz G. Inhibition of TEGDMA and HEMA-induced genotoxicity and cell cycle arrest by N-acetylcysteine. Dent Mater. 2007;23:688-95.

[7] Lee DH, Kim NR, Lim BS, Lee YK, Yang HC. Effects of

TEGDMA and HEMA on the expression of COX-2 and iNOS in cultured murine macrophage cells. Dent Mater. 2009;25:240-6

[8] Chang HH, Chang MC, Huang GF, Wang YL, Chan CP, Wang TM, Lin PS, Jeng JH. Effect of triethylene glycol dimethacrylate on the cytotoxicity, cyclooxygenase-2 expression and prostanoids production in human dental pulp cells. Int Endod J. 2012;45:848-58.

[9] Eckhardt A, Harorli T, Limtanyakul J, Hiller KA, Bosl C, Bolay C, Reichl FX, Schmalz G, Schweikl H. Inhibition of cytokine and surface antigen expression in LPS-stimulated murine macrophages by triethylene glycol dimethacrylate. Biomaterials. 2009;30:1665-74.

[10] Bølling AK, Samuelsen JT, Morisbak E, Ansteinsson V, Becher R, Dahl JE, Mathisen GH. Dental monomers inhibit LPS-induced cytokine release from the macrophage cell line RAW264.7. Toxicol Lett. 2013;216:130-8

[11] Izumi T, Kobayashi I, Okamura K, Sakai H. Immunohistochemical study on the immunocompetent cells of the pulp in human non-carious and carious teeth. Arch Oral Biol. 1995;40:609-14.

[12] Nakanishi T, Takahashi K, Hosokawa Y, Adachi T, Nakae H, Matsuo T. Expression of macrophage inflammatory protein 3alpha in human inflamed dental pulp tissue. J Endod. 2005;31:84-7.

[13] Novak ML, Koh TJ. Macrophage phenotypes during tissue repair. J Leukoc Biol. 2013;93:875-81

[14] Takei E, Shigetani Y, Yoshiba K, Hinata G, Yoshiba N, Okiji T. Initial transient accumulation of M2 macrophage-associated

molecule-expressing cells after pulpotomy with mineral trioxide aggregate in rat molars. J Endod. 2014;40:1983-8.

[15] Park HC, Quan H, Zhu T, Kim Y, Kim B, Yang HC. The effects of M1 and M2 macrophages on odontogenic differentiation of human dental pulp cells. J Endod. 2017;43:596-601.

[16] Heil TL, Volkmann KR, Wataha JC, Lockwood PE. Human peripheral blood monocytes versus THP-1 monocytes for in vitro biocompatibility testing of dental material components. J Oral Rehabil. 2002;29:401-7.

[17] Osman-Tolga H, Uusuf-Ziya B, Zuhal A, Abdulgani T. Cytotoxic effects of TEGDMA on THP-1 cells *in vitro*. Med Oral Patol Oral Cir Bucal 2009;14:489-93

[18] Izumi F, Atsushi T, Mitsuaki M, Hiroyuki M, Nobuhiko Y. Sensitization potential of dental resins: 2-hydroxyethyl methacrylate and its water-soluble oligomers have immunostimulatory effects. PLoS One. 2013;8:82540

[19] Santerre JP, Shajii L, Tsang H. Biodegradation of commercial dental composites by cholesterol esterase. J Dent Res. 1999;78:1459-68

[20] Spahl W, Budzikiewicz H, Geurtsen W. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. J Dent 1998;26:137-145

[21] Engelmann J, Leyhausen G, Leibfritz D, Geurtsen W. Metabolic effects of dental resin components in vitro detected by NMR spectroscopy. J Dent Res 2001;80:869-875

[22] Geurtsen W, Leyhausen G. Chemical-Biological interactions of

the resin monomer triethyleneglycol-dimethacrylate (TEGDMA). J Dent Res. 2001;80:2046-50.

[23] Ansteinsson V, Kopperud HB, Morisbak E, Samuelsen JT. Cell toxicity of methacrylate monomers-The role of glutathione adduct formation. J Biomed Mater Res A. 2013;101:3504-10.

[24] Circu ML, Aw TY. Glutathione and modulation of cell apoptosis. Biochim Biophys Acta. 2012;1823:1767-77.

[25] Nocca G, De Palma F, Minucci A, De Sole P, Martorana GE, Callà C, Morlacchi C, Gozzo ML, Gambarini G, Chimenti C, Giardina B, Lupi A. Alterations of energy metabolism and glutathione levels of HL-60 cells induced by methacrylates present in composite resins. J Dent. 2007;35:187-94.

[26] Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. Biomaterials 2013;34:4555-63.

[27] Ansteinsson V, Solhaug A, Samuelsen JT, Holme JA, Dahl JE. DNA-damage, cell-cycle arrest and apoptosis induced in BEAS-2B cells by 2-hydroxyethyl methacrylate (HEMA). Mutat Res. 2011;723:158-64.

[28] Geurtsen W. Biocompatibility of resin-modified filling materials. Crit Rev Oral Biol Med. 2000;11:333-55

[29] Reichl FX, Durner J, Hickel R, Kunzelmann KH, Jewett A, Wang MY, Spahl W, Kreppel H, Moes GW, Kehe K, Walther U, Forth W, Hume WR. Distribution and excretion of TEGDMA in guinea pigs and mice. J Dent Res. 2001;80:1412-5.

[30] Katsuno K, Manabe A, Itoh K, Hisamitsu H, Wakumoto S, Nakayama S, Yoshida T. A delayed hypersensitivity reaction to dentine primer in the guinea-pig. J Dent. 1995:23:295-9.

[31] Kallus T, Mjir I. Incidence of adverse effects of dental materials. Scand I Dent Res. 1991;99:236-40.

[32] Hallstrbm U. Adverse reaction to a fissure sealant: report of case. ASDC J Dent Child. 1993;60:143-6.

[33] Kanerva L, Henriks-Eckerman M-L, Estlander T, Jolanki R, Tarvainen K. Occupational allergic contact dermatitis and composition of acrylates in dental bonding systems. J Eur head Derm Venereol 1994;3:157-169.

[34] Bjϕrndal I, Darvann T. A light microscopic study of odontoblastic and non-odontoblastic cells involved in tertiary dentinogenesis in well defined cavitated carious lesions. Caries Res 1999;33:50-60

[35] Cooper PR, Holder MJ, Smith AJ. Inflammation and regeneration in the dentin-pulp complex: a double-edged sword. J Endod. 2014;40:46-51

[36] Smith AJ, Cassidy N, Perry H, Bègue-Kirn C, Ruch JV, Lesot H. Reactinary dentinogenesis. Int J Dev Biol 1995;39:273-80

[37] Bayers MR, Schatteman DC, Bothwell M. Multiple functions for NGF receptor in developing, aging and injured rat teeth are suggested by epithelial, mesenchymal and neural immunoreactivity. Development 1990;109:461-71

[38] Farges JC, Carrouel F, Keller JF, Baudouin C, Msika P, Bleicher

F, Staquet MJ. Cytokine production by human odontoblast-like cells upon Toll-like receptor-2 engagement. Immunobiology. 2011;216:513-7.

[39] Janeway CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197-216

[40] Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. 2010;32:593-603

[41] Solinas G, Gernamo G, Mantovani A, Allavena P. Tumor-asociated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol. 2009;86:1065-73

[42] Mosmann TR Cokkman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-73

[43] Abramson SL, Gallin JI. IL-4 inhibits superoxide production by human mononuclear phagocytes. J Imunol. 1990;144:625-30

[44] Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J Exp Med. 2007;204:1057-69

[45] Tjiu JW, Chen JS, Shun CT, Lin SJ, Liao YH, Chu CY, Tsai TF, Chiu HC, Dai YS, Inoue H, Yang PC, Kuo ML, Jee SH. Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. J Invest Dermatol. 2009;129:1016-25.

[46] Park HC, Quan H, Yang HC. Effects of phosphatidylserine-containing liposomes on odontogenic differentiation

of human dental pulp cells. Dent Mater J. 2017;36:76-81.

[47] Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. Immunity. 2014;41:21-35.

[48] Korolnek T, Hamza I. Macrophages and iron trafficking at the birth and death of red cells. Blood. 2015;125:2893-7.

[49] Lavin Y, Mortha A, Rahman A, Merad M. Regulation of macrophage development and function in peripheral tissues. Nat Rev Immunol. 2015;15:731-44.

[50] Corliss BA, Azimi MS, Munson JM, Peirce SM, Murfee WL. Macrophages: an Inflammatory Link Between Angiogenesis and Lymphangiogenesis. Microcirculation. 2016;23:95-121.

[51] Okiji T, Kawashima N, Kosaka T, Matsumoto A, Kobayashi C, Suda H. An immunohistochemical study of the distribution of immunocompetent cells, especially macrophages and Ia antigen-expressing cells of heterogeneous populations, in normal rat molar pulp. J Dent Res. 1992;71:1196-202.

[52] Bolling AK, Solhaug A, Morisbak E, Holme JA, Samuelsen JT. The dental monomer hydroxyethyl methacrylate (HEMA) counteracts lipopolysaccharide-induced IL-1β release-Possible role of glutathione. Toxicol Lett. 2017;270:25-33.

[53] Orsolic N, Kunstic M, Kukolj M, Gracan R, Nemrava J. Oxidative stress, polarization of macrophages and tumour angiogenesis: efficacy of caffeic acid. Chem Biol Interact. 2016;256:111-24.

[54] Lefeuvre M, Amjaad W, Goldberg M, Stanislawski L. TEGDMA

induced mitochondrial damage and oxidative stress in human gingival fibroblasts. Biomaterials. 2005;26:5130 - 7.

-국문초록-

Triethylene glycol dimethacrylate와 hydroxyethyl methacrylate가 대식세포의 분극에 미치는 영향

서울대학교 대학원 치의과학과 치과생체재료과학 전공

(지도교수 양 형 철)

김 인 숙

희석용 레진 모노머인 triethylene glycol dimethacrylate (TEGDMA)와 친수성 레진 모노머인 hydroxyethyl methacrylate (HEMA)는 치과용 충전재와 상아질 접착제 등에 빈번히 사용되며, 불완전한 중합으로 인해 미반응 모노머가 구강 환경에 용출되어 염증반응 및 면역반응을 유도할 수 있다. 본 연구에서는

TEGDMA와 HEMA가 사람 대식세포 세포주인 THP-1의 분극에 미치는 영향을 관찰하였다.

THP-1 세포를 세포독성이 나타나지 않은 농도에서 레진 모노머로 48시간 동안 처리한 후, M1과 M2 마커인 CD86과 CD206의 발현을 flow cytometry로 분석하였다. 대식세포의 분극에 미치는 영향을 관찰하기 위하여, 레진 모노머 처리는 대식세포 분극유도물질과 동시에, 혹은 레진 모노머로 전처리하여 M1, M2 분극화를 유도하였다. 분극화 정도는 qPCR에 의한 CD86, CD206의 mRNA 정량과 TNF-α 및 TGF-β의 효소면역측정 분석으로 관찰하였다. 또한, 분극화에 의한 세포 형태변화를 현미경 하에서 관찰하였다. 통계분석은 일원분산분석을 사용하였다.

TEGDMA (1 mmol/L)와 HEMA (2 mmol/L)는 THP-1 세포에서 CD86 및 CD206 발현을 유도하지 않았고, 오히려 분극화 유도물질의 동시처리에서 CD86과 CD206의 발현을 억제하였다. 발현 억제 효과는 mRNA의 전사 단계에서 나타났다. 또한, 레진 모노머는 M1, M2 유도 세포의 TNF-α 및 TGF-β 생성을 저해하였으며, 분극과 동반하는 세포 형태의 변화도 레진 모노머에 의해 억제되었다. 그러나, 레진 모노머의 전처리에 의한 일시적인 접촉은 분극화 유도물질에 의한 CD86과 CD206의 발현에 영향을 주지 않았다. 즉, 레진 모노머의 분극화 억제는 가역적으로 판단된다.

결론적으로, TEGDMA와 HEMA는 대식세포의 M1, M2 분극을 전사 단계에서 저해하였으며, 저해 효과는 세포배양액에서 레진 모노머의 제거와 함께 소실된다.

주요어 : triethylene glycol dimethacrylate, hydroxyethyl methacrylate, macrophage, polarization

학　　번: 2011-31194